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Research paper

Stress-induced aggression in heterozygous TPH2 mutant mice is associated with alterations in serotonin turnover and expression of 5-HT6 and AMPA subunit 2A receptors

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ABSTRACT

Background: The contribution of gene-environment interactions that lead to excessive aggression is poorly understood. Environmental stressors and mutations of the gene encoding tryptophan hydroxylase-2 (TPH2) are known to influence aggression. For example, TPH2 null mutant mice (Tph2−/−) are naturally highly aggressive, while heterozygous mice (Tph2+/−) lack a behavioral phenotype and are considered endophenotypically normal. Here we sought to discover whether an environmental stressor would affect the phenotype of the genetically 'susceptible' heterozygous mice (Tph2+/−).

Methods: Tph2+/− male mice or Tph2+/+ controls were subjected to a five-day long rat exposure stress paradigm. Brain serotonin metabolism and the expression of selected genes encoding serotonin receptors, AMPA receptors, and stress markers were studied.

Results: Stressed Tph2+/− mice displayed increased levels of aggression and social dominance, whereas Tph2+/+ animals became less aggressive and less dominant. Brain tissue concentrations of serotonin, its precursor hydroxytryptophan and its metabolite 5-hydroxyindoleacetic acid were significantly altered in all groups in the prefrontal cortex, striatum, amygdala, hippocampus and dorsal raphe after stress. Compared to non-stressed animals, the concentration of 5-hydroxytryptophan was elevated in the amygdala though decreased in the other brain structures. The overexpression of the AMPA receptor subunit, GluA2, and downregulation of 5-HT6 receptor, as well as overexpression of c-fos and glycogen-synthase-kinase-3β (GSK3-β), were found in most structures of the stressed Tph2+/− mice.

Limitations: Rescue experiments would help to verify causal relationships of reported changes.

Conclusions: The interaction of a partial TPH2 gene deficit with stress results in pathological aggression and molecular changes, and suggests that the presence of genetic susceptibility can augment aggression in seemingly resistant phenotypes.

1. Introduction

Aberrant social behaviours, particularly aggression, violence and antisocial behavior, often accompany neurodevelopmental (De Giacomo et al., 2016) and neurodegenerative (Levenson et al., 2014) conditions, as well as depression (Knox et al., 2000) and stress-related disorders (Van Voorhees et al., 2014). Aggressive behaviour during major depression is associated with an enhanced risk of suicide

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(Gvion & Apter, 2011). Pathological aggression is considered to be a result of complex gene-environment interactions dependent on biological, psychological and social factors (Lesch & Merschdorf, 2000; Lesch et al., 2012). Persistent life stress and mutations of the gene encoding tryptophan hydroxylase-2 (TPH2), the predominant enzyme required for neuronal 5-HT synthesis, are established as causes of excessive aggression (Liu, 2004; Perez-Rodriguez et al., 2010; Jager et al., 2018). However, the contribution of the interaction in this pathological behavior is still poorly understood.

Aberrant 5-HT metabolism is an established factor for emotional imbalance and increased aggressiveness (Linnoila & Virkkunen, 1992; Lesch, 2005). Compromised TPH2 activity may contribute to the manifestation of these traits. Human data suggest that there is a relationship between TPH2 single nucleotide polymorphisms (SNPs) and negative emotionality (Strobel et al., 2007), as well as deficits in cognitive control and emotion regulation (Waider et al., 2011). SNPs in TPH2 have also shown to be associated with an increased risk of suicidal behavior (Zhang et al., 2010) and incidence of depression (Wigner et al., 2018). In mice with complete genetic inactivation of TPH2 function (Tph2−/−), a lack of brain 5-HT results in increased aggression in the absence of an environmental stressor (Angoa-Pérez et al., 2012). Also evident in these animals are impulsivity, anxiety-related behavior, and an increased fear response, which are all behavioural features that often accompany excessive aggression in animals (Coccaro et al., 2011; Neumann et al., 2009; Agis-Balboa et al., 2009). For example, aggressive Tph2−/− mice displayed signs of compulsive behaviours in the nestlet shred and the marble burying tests (Angoa-Pérez et al., 2012), and elevated scores of anxiety-like behavior in a battery of tests (Angoa-Pérez et al., 2012; Mosienko et al., 2012; Lesch et al., 2012; Gutknecht et al., 2015). Increased conditioning of the response was found in Tph2−/− mice in a contextual fear conditioning paradigm (Lesch et al., 2012) and in auditory conditioning (Gutknecht et al., 2015). Parameters of aggression could be attenuated by administration of the 5-HT precursor, 5-hydroxytryptophan (5-HTP) (Angoa-Pérez et al., 2012; Mosienko et al., 2012).

The changes in aggressive and other emotional behaviors in the Tph2−/− are believed to be owing to the lack of 5-HT in the brain of the mutants, as immunofluorescent labeling confirmed the absence of specific 5-HT immunoreactivity in the raphe neurons and other brain regions of Tph2−/− mice (Gutknecht et al., 2008). Tph2−/− mice exhibit other neurochemical and molecular abnormalities, such as increased 5-HT1a and 5-HT1b receptor abundance and binding capacity (Gutknecht et al., 2012; Araragi et al., 2013) and a reduction in the density of GABAergic interneurons in the basolateral amygdala (Waider et al., 2019).

The 5-HT system is known to be critical in the regulation of aggression in both animals and humans. Impulsivity and high levels of aggression were found to correlate with low cerebrospinal fluid concentrations of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA), in humans and nonhuman primates, and reduced 5-HT levels or turnover in the brain of rhesus monkeys (Lesch & Merschdorf, 2000; Lesch, 2005). Genetic evidence for a role of 5-HT in aggression comes from studies on other mutant mice besides the Tph2−/−, which display altered 5-HT concentrations or metabolism (Tenpenny & Commons, 2017). For example, mice with a knockout for voltage-dependent N-type Ca(2+) channels display altered concentrations of brain 5-HT associated with excessive aggressive behaviors (Kim et al., 2009). Mice with partial or complete general transcription factor II-I repeat domain-containing protein 1 deficiency have altered 5-HT levels in the brain that correlate with changes in aggressiveness (Young et al., 2008). However, partial neuronal deficit of 5-HT in unchallenged animals does not appear to lead to any behavioural changes. This is the case for the Tph2+/− mice, which show a 20% reduction of brain 5-HT compared to naive controls (Mosienko et al., 2012).

Stress is known to potentiate brain 5-HT deficiency leading to a reduction in neuronal 5-HT in mice (Sachs et al., 2013), and this reduction appears to trigger aggression (Vogel & Schwabe, 2019). Chronic and acute stress are considered the major environmental factors that interact with genetic predispositions to aggressive and violent behavior (Conway et al., 2012). In this work, we sought to test the hypothesis of whether stress exposure combined with the partial genetic deficit of Tph (Tph2+/−) might result in a 'double-hit' effect on 5-HT levels in mice and induce aggression typical of the naïve Tph2−/− mice.

To explore the effect of a 'double-hit' on behaviour, Tph2+/− male mice were subjected to predation stress, which has been shown to induce marked behavioural, cellular and molecular abnormalities (Strekalova et al., 2018; Vignisse et al., 2017). Social behavior, brain 5-HT metabolism, and gene expression of earlier established molecular markers of stress-induced aggression were evaluated. Our target genes were selected on the basis of our previous studies that revealed an overexpression of AMPA receptor subunit GluA2, glycogen-synthase-kinase-3β (GSK-3β) and downregulation of 5-HT6 receptor in ultrasound-induced aggression in BALB/c mice (Gorlova et al., 2019; Pavlov et al., 2019; Costa-Nunes et al., 2020).

2. Materials and methods

2.1. Animals

We used 10-12-week-old Tph2+/− male mice, and their wildtype littermates as controls, which were bred and genotyped in the facilities at New University of Lisbon as previously described elsewhere (Gutknecht et al., 2015). 12-week-old male CD1 mice were used as intruders for a resident-intruder test and 2-5-month-old Wistar rats (Charles River, Janvier, France) were used for predator stress. Tph2+/− and Tph2+/+ mice were housed individually, while CD1 mice and rats were housed in groups of five. Animals were kept under controlled laboratory conditions (22 ± 1°C, 55% humidity, food and water ad libitum, lights on: 21:00 h). Studies were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals (permission 0421/000/000/2013 issued by Ethical Committee of the New University of Lisbon).

2.2. Study outline

Groups of mice were balanced according to their body weight, and the latency to attack was evaluated in a baseline resident-intruder test (Couch et al., 2016, Strekalova et al., 2018; *see below*). Mutants and wildtype controls were subjected to a daily rat-exposure stress paradigm for 5 days (Vignisse et al., 2017; *see below*) and starting 24 h thereafter were tested in a resident-intruder test during five consecutive days, along with non-stressed groups. The unstressed group was not exposed to a rat or to any other alteration in the environment, other than daily handling; the unstressed group was also housed in a separate room. After 24 hours, mice were killed and their brains were isolated and dissected for subsequent HPLC and RT-PCR assays (Fig. 1). In both *in vivo* and *in vitro* assays seven animals per group were used unless otherwise stated in figure legends.

2.3. Predation stress

Mice were introduced into a transparent glass cylinder (15cm high x 8cm diameter) and placed into the rat cage as described elsewhere (Costa-Nunes et al., 2014; Vignisse et al., 2017). 15-h exposure was performed between 18:00 and 9:00 for 5 consecutive nights. Mice had free access to food and water in their home cages between the stress sessions.

2.4. Resident-Intruder test

The resident-intruder test procedure was carried out over 5

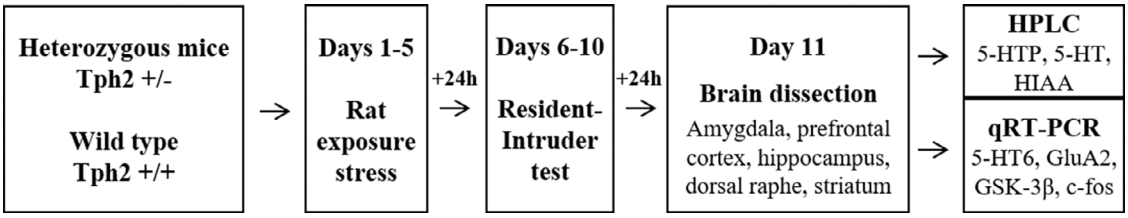


Fig. 1. Experiment design.
Tph2+/+ and Tph2+/− mice were subjected to rat exposure stress for 5 consecutive nights, studied in 5 consecutive days in the resident-intruder test and killed. Their brained were dissected for subsequent quantitative reverse transcription polymerase chain reaction (qRT-PCR) and high performance liquid chromatography (HPLC) assays.

consecutive days (Strekalova et al., 2004, 2018; Costa-Nunes et al., 2014; Couch et al., 2013, 2016). Mice were placed individually in an observation cage (30 × 60 × 30 cm) for 30 min, after which a CD1 mouse was introduced. During the first 4 minutes, mice were separated by a transparent wall with holes that was removed for the following 4 minute period. The latency to follow and attack, the number and total duration of attacks and followings were recorded, to score aggressive and dominant behavior, in 2-min intervals (Couch et al., 2016; Gorlova et al., 2019).

2.5. Sacrifice and tissue collection

Mice were terminally anaesthetized with an intraperitoneal injection of sodium pentobarbitone. The left ventricle was perfused with 10 ml ice-cold saline and brains were dissected (Couch et al., 2013; Strekalova et al., 2016). The prefrontal cortex, striatum, amygdala, hippocampus and dorsal raphe were isolated and stored at -80°C as described elsewhere (Gorlova et al., 2019).

2.6. High Performance Liquid Chromatography (HPLC)

The concentrations of 5-HT, its precursor 5-HTP and metabolite 5-HIAA were measured in the above-mentioned brain areas using HPLC with electrochemical detection using the method of Waider et al. (2017) (*see Supplementary material*). 5-HT turnover as a ratio of 5-HT/5-HIAA was calculated.

2.7. Quantitative reverse transcription polymerase chain reaction analysis (qRT-PCR)

Total mRNA was isolated with TRI Reagent (Invitrogen, Carlsbad, CA, USA) and converted into cDNA using random primers and Superscript III transcriptase (Invitrogen, Carlsbad, CA, USA); qRT-PCR was performed using a SYBR Green master mix (5 µl, Bio-Rad Laboratories, Philadelphia, PA, USA) in triplicate (*see Supplementary material and Suppl. Table 1*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the reference gene, and data were normalized to its expression and expressed as relative-fold changes compared to control values (Couch et al., 2016; Gorlova et al., 2019; Strekalova et al., 2016).

2.8. Statistical analysis

Data were analyzed using GraphPad Prism version 6.0 for Windows (San Diego, CA). The data were analyzed for normality using Shapiro-Wilk test and for the presence of outliers. Data that were not normally distributed were subject to a Kruskal-Wallis test. Normally distributed data were analyzed with two-way ANOVA. Levene's test was used to verify equal variance, and, where the data was homoscedastic, they were then analyzed with a post-hoc Tukey's test; if the variances were heteroscedastic, a Bonferroni test was used. Level of confidence set at 95% ($p < 0.05$).

3. Results

3.1. Increased aggressive behavior in stressed Tph2+/− but not Tph2+/+ mice

A Kruskal-Wallis test revealed significant differences between groups in the average latency to attack and the average number and duration of attacks ($H = 10.21$, $p = 0.0169$; $H = 9.673$, $p = 0.044$; $H = 8.93$, $p = 0.0486$, respectively). In comparison to the non-stressed Tph2+/− group, stressed mutants displayed a decreased latency to attack ($p = 0.0169$; Fig. 2A) and increases in the number and duration of attacks ($p = 0.023$ and $p = 0.044$, respectively; Fig. 2B,C). Together, all these measures indicate that aggression is increased in Tph2+/− mice that are subject to stress.

Analysis of the daily scores of the aggressive behaviors also revealed that the stress significantly elevated signs of aggression during the 5-day testing period. There were significant group differences in the latency to attack on days 3 and 5 ($H = 8.268$, $p = 0.0408$ and $H = 8.501$, $p = 0.0367$, respectively, Kruskal-Wallis test), where the stressed Tph2+/− mice exhibited a decrease in latency in comparison with stressed Tph2+/+ ($p = 0.0425$ and $p = 0.0417$, respectively; Fig. 2D). No significant group differences in this behavior were found on days 1, 2 and 4 ($H = 5.926$,$p = 0.1153$; $H = 5.881$, $p = 0.1176$ and $H = 7.059$, $p = 0.07$, respectively). Significant differences in the number of attacks between the groups were present on days 1 and 5 ($H = 8.51$, $p = 0.0366$ and $H = 9.048$, $p = 0.0287$, respectively, Kruskal-Wallis test), where significantly higher scores were displayed by stressed Tph2+/− than Tph2+/+ mice at the later time point ($p = 0.0309$; Fig. 2E). No significant differences between the groups were revealed on days 2-4 ($H = 3.857$, $p = 0.2773$; $H = 6.88$, $p = 0.0728$ and $H = 5.714$, $p = 0.1264$, respectively). The total duration of attacks was significantly different between the groups on the last day of the resident-intruder test ($H = 11.89$, $p = 0.0078$; Kruskal-Wallis test), when stressed Tph2+/− mice displayed increased duration of attacks in comparison with their non-stressed counter-partners ($p = 0.0329$) and stressed Tph2+/+ animals ($p = 0.0193$; Fig. 2F). No group changes was revealed on days 1-4 ($H = 6.18$, $p = 0.1032$; $H = 4.57$, $p = 0.2061$; $H = 5.665$, $p = 0.1981$ and $H = 5.308$, $p = 0.1506$, respectively).

Analysis of aggression patterns during the 4-min resident-intruder test showed no significant differences within the groups in the number of attacks in the first and second half of the exposure (1-2 min or 3-4 min) ($H = 5.34$, $p = 0.0912$ and $H = 4.204$, $p = 0.1213$, respectively; Kruskal-Wallis test Fig. 2G). However, there were significant group differences in the duration of attacks during minutes 1-2 ($H = 9.32$, $p = 0.0351$); the stressed Tph2+/− mice exhibited more attacks than the non-stressed mutants ($p = 0.0206$) and the stressed Tph2+/+ group ($p = 0.0211$; Fig. 2H). No significant group differences in this aggression measure were found for minutes 3-4 of the test ($H = 6.12$, $p = 0.103$). Thus, unlike other groups, stressed Tph2+/− mice displayed an increase of aggressive behavior in the first few minutes of the resident-intruder interaction.

Together, Tph2+/− mice displayed elevated aggression scores

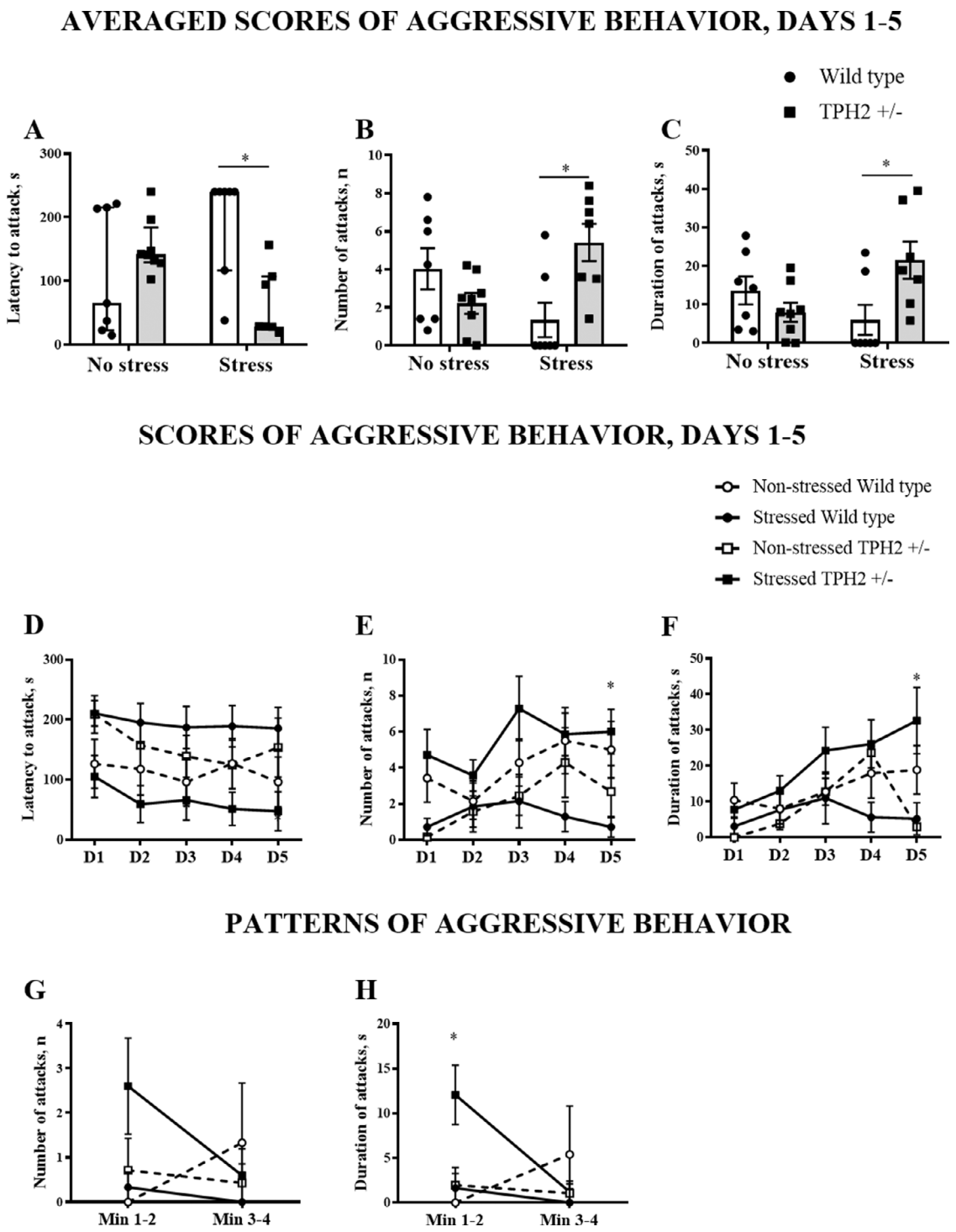


Fig. 2. Opposing effects of predator stress on aggressive behavior of Tph2+/+ and Tph2+/− mice.
In comparison with stressed controls, stressed Tph2+/− mice exhibited asignificantly **(A)** decreased latency to attack, **(B)** increased number of attacks and **(C)** duration of attacks, averaged for 5 days. Stressed Tph2+/+ mice displayed significantly fewer numbers of attacks than non-stressed Tph2+/+. Daily analysis revealed that stressed Tph2+/− mice exhibited **(D)** decreased latency to attack in comparison with stressed Tph2+/+ mice on day 3 and on day 5 and **(E)** increased number of attacks on day 5. **(F)** On day 5 stressed Tph2+/− mice displayed increased duration of attacks in comparison with non-stressed Tph2+/− and stressed Tph2+/+ mice. **(G)** No differences were revealed in number of attacks during minutes 1-2 and 3-4. **(H)**Tph2+/− mice displayed significantly prolonged duration of attacks after stress in comparison with non-stressed Tph2+/− mice and stressed Tph2+/+ mice during minutes 1-2, but not 3-4. *$p < 0.05$, Kruskal-Wallis test. Tph2+/+ No stress, n=7, Tph2+/+ Stress, n=7, Tph2+/− No stress, n=8, Tph2+/− Stress, n=7. Bars are Median with interquartile range.

following exposure to stress, while wildtype mice exhibited the opposite behavioural trend. These contrasting behaviours were observed during daily scoring of aggressive behavior in the five-day test and were more evident after averaging across entire period of testing. Importantly, stressed Tph2+/− mice displayed a remarkably distinct aggression pattern compared to the other groups in that they showed an increase in aggressive behavior in the first minutes of the social contact.

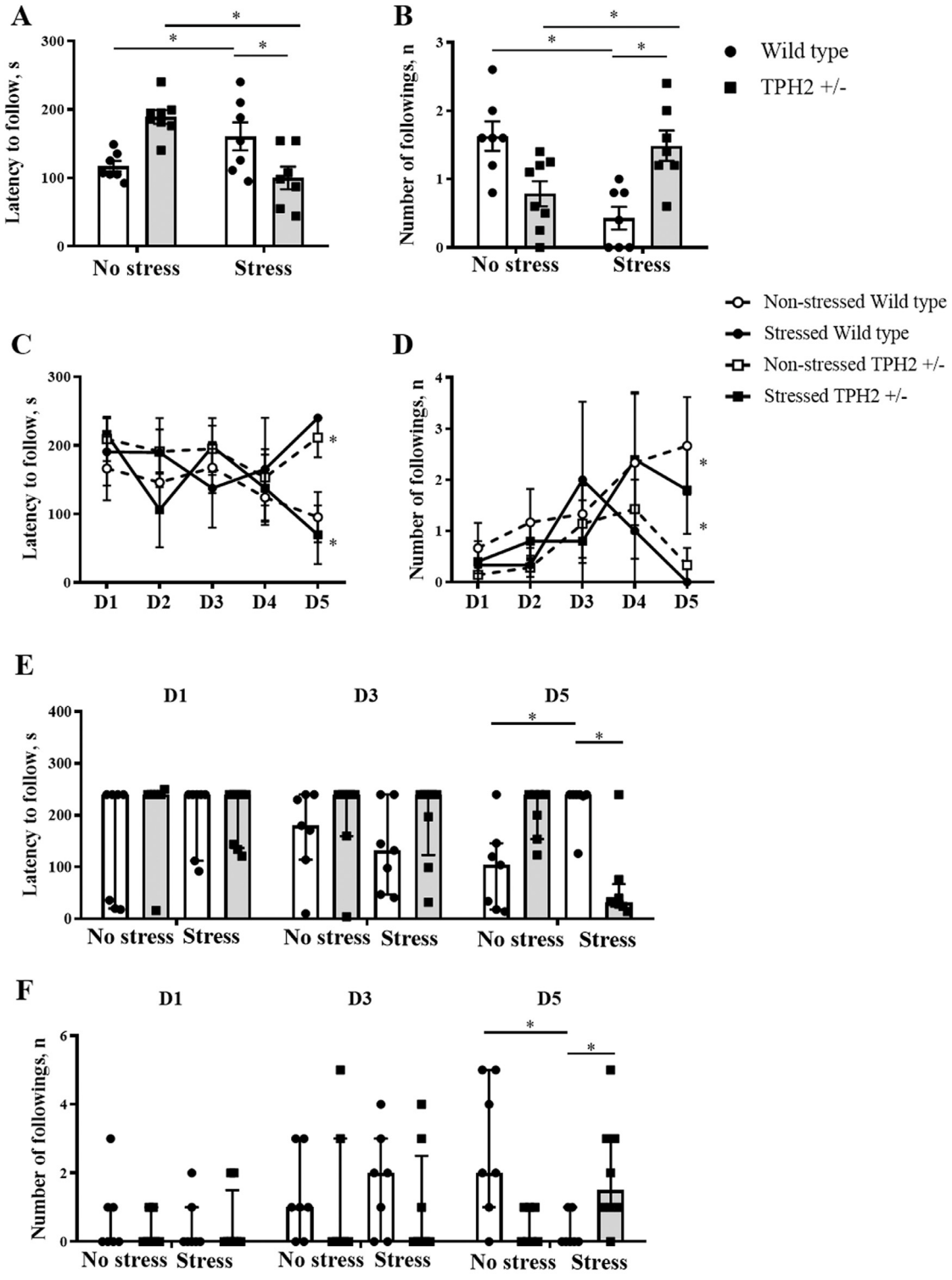


Fig. 3. Differential changes in dominant-like behavior of Tph2+/+ and Tph2+/− mice exposed to stress.
(A) In comparison with non-stressed Tph2+/− mice and stressed Tph2+/+ group, stressed Tph2+/− mice displayed a significantly decreased average latency to follow and **(B)** elevated average number of follows, while stressed Tph2+/+ exhibited the opposite changes in these parameters compared to non-stressed Tph2+/+ mice. **(C,E)** No significant differences were found between groups in latency to follow and **(D,F)** in number of followson days 1-4. Stressed Tph2+/− mice exhibited lower latency to follow and higher number of follows than non-stressed Tph2+/− mice and stressed Tph2+/+ on day 5. At the same time, stressed Tph2+/+ displayed augmented latency to follow and decreased number of follows in comparison with non-stressed Tph2+/+. *$p < 0.05$, Kruskal-Wallis test, two-way ANOVA and post hoc Tukey's test or Bonferroni test. Tph2+/+ No stress, n=7, Tph2+/+ Stress, n=7, Tph2+/− No stress, n=8, Tph2+/− Stress, n=7. Bars are Mean ± SEM (A, B) or Median with interquartile range (E, F).

3.2. Increased dominant-like behavior in Tph2+/− mice and decreased signs of dominancy in Tph2+/+ animals after stress exposure

Changes in aggressive behavior were also accompanied by alterations in social dominance as evidenced by changes in following behaviour. Significant main effects of stress ($F_{3,28}=5.23$, $p=0.0076$, two-way ANOVA) and genotype ($F_{3,28}=6.19$, $p=0.0095$), as well as stress and genotype interaction ($F_{3,28}=19$, $p=0.0004$) on the average latency to 'follow' were found. Stressed Tph2+/− mice demonstrated lower latency to follow than non-stressed Tph2+/− mice ($p=0.0458$, Bonferroni's test) and stressed Tph2+/+ animals ($p=0.0012$, Fig. 3A). By contrast, Tph2+/+ animals exhibited a prolonged averaged latency to follow in comparison with their non-stressed counterparts ($p=0.0097$).

ANOVA revealed significant main effects of stress ($F_{3,28}=4.99$ $p=0.046$, two-way ANOVA) and genotype ($F_{3,28}=5.08$, $p=0.038$), and stress and genotype interaction ($F_{3,28}=13.1$, $p=0.0021$) on the average number of follows. Stressed Tph2+/− mice displayed higher scores of this behavior than non-stressed Tph2+/− mice ($p=0.034$, Tukey's test) and stressed Tph2+/+ animals ($p=0.0405$; Fig. 3B). The latter group of animals had lower number of followings than non-stressed controls ($p=0.0386$).

A Kruskal-Wallis test revealed significant differences between groups in latency to follow and number of follows on day 5 ($H=7.497$, $p=0.0405$ and $H=8.544$, $p=0.013$, respectively), stressed Tph2+/− mice displayed a decrease of the former and an increase of the latter parameter in comparison with stressed Tph2+/+ group ($p=0.0238$ and $p=0.0473$, respectively; Fig. 3C-F), which exhibited opposing changes in this behaviour in comparison with the non-stressed controls ($p=0.0182$ and $p=0.0267$). No group significant group differences were found on days 1-4 in the latency to follow ($H=1.289$, $p=0.7317$; $H=3.101$, $p=0.3763$; $H=2.209$, $p=0.5303$ and $H=0.2726$, $p=0.9651$, respectively) and number of follows ($H=0.8805$, $p=0.8301$; $H=2.045$, $p=0.5631$; $H=1.987$, $p=0.5752$ and $H=0.7926$, $p=0.8512$, respectively).

These data are evidence of the increase in dominant-like features of stressed Tph2+/− mice, further supporting the characteristic of elevated aggressiveness in these mice. At the same time, Tph2+/+ mice showed a reduction of dominant-like behaviors after the stress exposure.

3.3. Stress-induced alterations of concentrations of 5-HT, its precursor and metabolite in the brain of Tph2+/− and Tph2+/+ mice

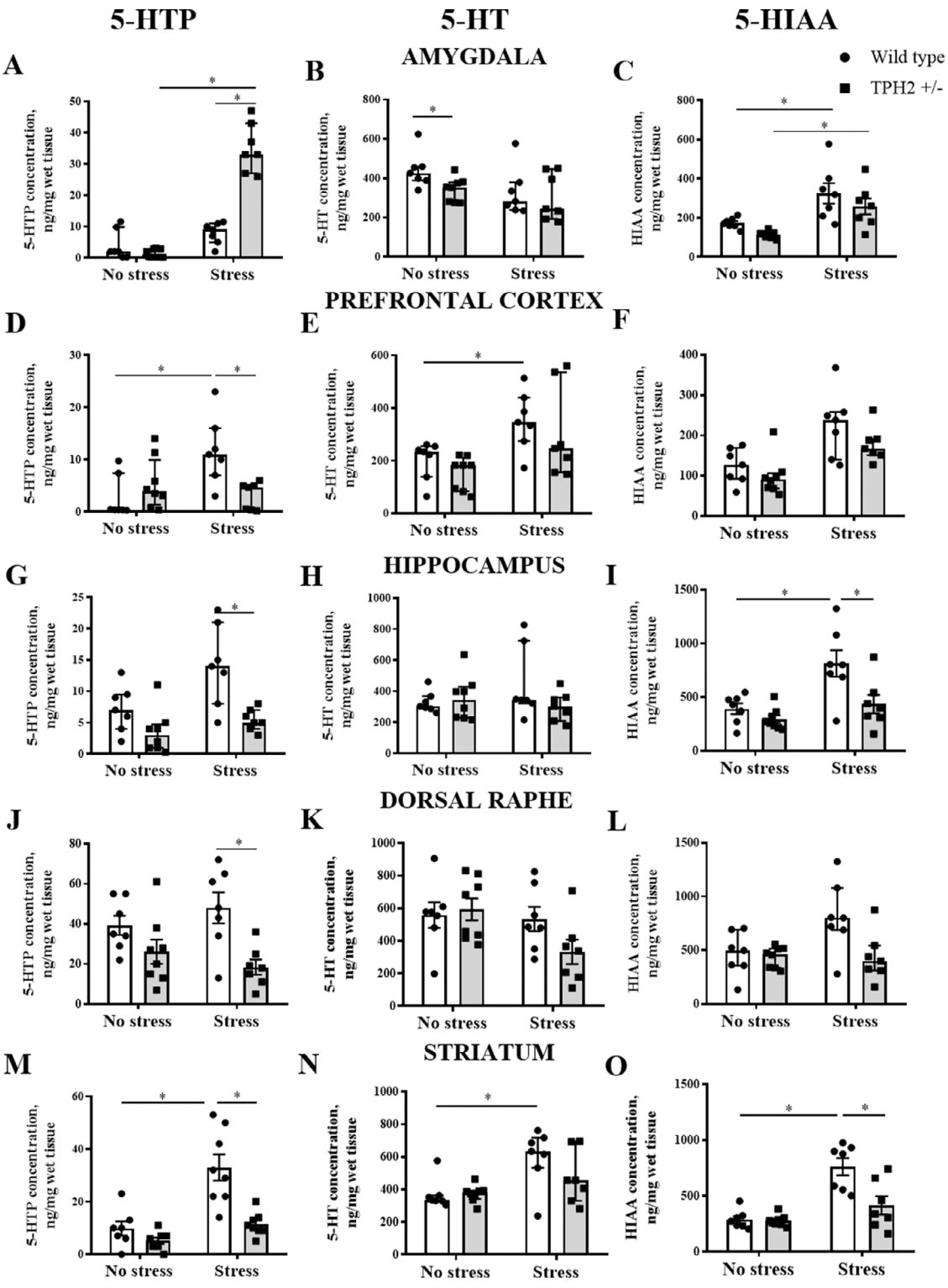
We observed significant group differences in 5-HTP concentration in the amygdala ($H=21.03$, $p=0.0001$, Kruskal-Wallis test), which was elevated in stressed Tph2+/− mice in comparison with non-stressed Tph2+/− mice and stressed Tph2+/+ mice ($p=0.0034$ and $p<0.0001$, respectively; Fig. 4A). A Kruskal-Wallis analysis showed significant differences between groups in the level of 5-HTP in the prefrontal cortex and the hippocampus ($H=11.17$, $p=0.0108$ and $H=13.39$, $p=0.0039$, respectively), significant decreases of this parameter in stressed Tph2+/− mice in comparison with stressed controls ($p=0.0415$ and $p=0.0023$, respectively; Fig. 4G). 5-HTP concentration in the prefrontal cortex was significantly higher in stressed Tph2+/+ mice than in non-stressed controls ($p=0.0122$; Fig. 4D). Two-way ANOVA revealed no significant effects of stress, and stress and genotype interaction for 5-HTP concentration in the dorsal raphe ($F_{3,28}=0.0751$, $p=0.7861$ and $F_{3,28}=1.12$, $p=0.2989$, respectively). There was a significant effect of genotype on 5-HTP concentration ($F_{3,28}=19$, $p=0.0002$), where stressed Tph2+/− mice exhibited its significant decrease in comparison with stressed Tph2+/+ controls ($p=0.025$, Tukey's test; Fig. 4J). For 5-HTP concentration in the striatum, there were significant effects of stress, genotype and their interaction ($F_{3,28}=133$, $p<0.0001$, $F_{3,28}=52.7$, $p<0.0001$ $F_{3,28}=7.79$, $p=0.0095$, respectively; two-way ANOVA). This parameter was significantly lower in the stressed Tph2+/− mice than in stressed controls ($p=0.0001$, Bonferroni test), while the latter group showed opposing changes in comparison with their non-stressed counter partners ($p<0.0001$; Fig. 4M). No further group differences in the 5-HTP concentration were found.

Concerning 5-HT, its concentration in the amygdala was significantly different between the groups ($H=9.399$, $p=0.0244$, Kruskal-Wallis test) and significantly decreased in non-stressed Tph2+/− mice in comparison with non-stressed Tph2+/+ animals ($p=0.0498$; Fig. 4B). Analysis with Kruskal-Wallis test demonstrated significant differences between groups in 5-HT concentration in the prefrontal cortex, but not in the hippocampus ($H=12.61$, $p=0.0056$ and $H=1.815$, $p=0.6118$, Kruskal-Wallis test; Fig. 4E,H). In the prefrontal cortex brain, 5-HT concentration was significantly elevated in the stressed Tph2+/+ mice in comparison with non-stressed controls ($p=0.043$; Fig. 4E). Two-way ANOVA revealed significant stress effects for 5-HT concentration in the dorsal raphe ($F_{3,28}=4.80$, $p=0.0377$), but no significant effect of genotype or stress and no genotype interaction ($F_{3,28}=0.672$, $p=0.4197$ and $F_{3,28}=1.66$, $p=0.2094$. respectively; Fig. 4K); no significant group differences were found. 5-HT concentration in the striatum was significantly different between groups ($H=12.61$, $p=0.0223$) and significantly increased in stressed Tph2+/+ mice in comparison with non-stressed controls ($p=0.0265$; Fig. 4N). No further group differences in the 5-HT levels were present.

5-HIAA concentration in the amygdala was significantly altered by stress ($F_{3,28}=24.5$, $p<0.0001$, two-way ANOVA) and genotype ($F_{3,28}=4.94$, $p=0.0349$), while no significant stress and genotype interaction was found ($F_{3,28}=0.0631$, $p=80.36$). This measure was significantly higher in both the stressed mutants and the Tph2+/+ mice than in respective non-stressed groups ($p=0.0146$ and $p=0.004$, respectively; Tukey's test Fig. 4C). Group comparisons for 5-HIAA concentration in the prefrontal cortex showed that there were significant differences ($H=14.07$, $p=0.0028$; Kruskal-Wallis test Fig. 4F). For hippocampal 5-HIAA concentration, we observed significant effects of stress and genotype ($F_{3,28}=4.96$, $p=0.0341$ and $F_{3,28}=6.46$, $p=0.0171$, respectively; two-way ANOVA), and no significant stress and genotype interaction ($F_{3,28}=0.642$, $p=0.4297$). 5-HIAA concentration was significantly higher in stressed Tph2+/+ mice than in the non-stressed group ($p=0.0107$, Tukey's test) and stressed Tph2+/− mice ($p=0.0429$; Fig. 4I). No significant effects of stress, genotype and their interaction were found for 5-HIAA concentration in the dorsal raphe ($F_{3,28}=0.0751$, $p=0.7861$, $F_{3,28}=4.16$, $p=0.0517$ and $F_{3,28}=2.84$, $p=0.1041$, respectively; two-way ANOVA Fig. 4L). Significant effects for 5-HIAA concentration in the striatum were found for stress ($F_{3,28}=23.6$, $p<0.0001$, two-way ANOVA), genotype ($F_{3,28}=11.8$, $p=0.0019$) and stress and genotype interaction ($F_{3,28}=10.4$, $p=0.0032$). Stressed Tph2+/+ mice displayed a significantly higher 5-HIAA concentration than non-stressed controls and stressed Tph2+/− mice ($p<0.0001$ and $p=0.0003$, respectively, Tukey's test; Fig. 4O). No further group differences were present. Together, predator stress was found to differentially alter the measures of 5-HT metabolism in Tph2+/− and Tph2+/+ mice in brain structures that were investigated.

3.4. Differential gene expression of 5-HT receptors and the AMPA receptor GluA2 subunit in Tph2+/− mice and Tph2+/+ controls: effects of stress

A Kruskal-Wallis test revealed no significant differences between groups in 5-HT6 mRNA concentration in the amygdala ($H=3.227$, $p=0.3579$; Fig. 5A). In the prefrontal cortex, there was significant effect of stress ($F_{3,28}=13.2$, $p=0.0019$, two-way ANOVA), but not of genotype, and there was no stress-genotype interaction ($F_{3,28}=1.85$, $p=0.1905$ and $F_{3,28}=1.40$, $p=0.2522$, respectively). In comparison with non-stressed Tph2+/− mice, stressed mutants exhibited a decrease in 5-HT6 mRNA ($p=0.0069$, Bonferroni test; Fig. 4C). Two-way ANOVA revealed significant effects of stress and genotype for 5-HT6



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mRNA concentration in the hippocampus ($F_{3,28}=12.9$, $p=0.0019$ and $F_{3,28}=7.26$, $p=0.0143$, respectively), but no significant stress-genotype interaction ($F_{3,28}=0.181$, $p=0.675$). A significant decrease of 5-HT6 mRNA concentration was found in stressed Tph2+/− mice in comparison with non-stressed mutants ($p=0.0289$, Tukey's test; Fig. 5E). Significant group differences in 5-HT6 mRNA concentration were found in the dorsal raphe and striatum ($H=12.8$, $p=0.0051$ and $H=9.587$, $p=0.0224$, respectively; Kruskal-Wallis test). Stressed Tph2+/− had significantly lower values of 5-HT6 mRNA than their non-stressed counter partners in both brain structures ($p=0.0297$ and $p=0.0112$, respectively; Fig. 5G) and were lower than controls, in the striatum ($p=0.0407$; Fig. 5I). There were no further group differences.

GluA2 mRNA concentration in the amygdala was not significantly affected by stress ($F_{3,28}=4.11$, $p=0.0548$), genotype ($F_{3,28}=0.448$, $p=0.5113$), or stress and genotype interaction ($F_{3,28}=1.36$, $p=0.2632$; Fig. 5B). GluA2 mRNA concentration in the prefrontal cortex was

Fig. 4. Stress-induced alterations of brain concentrations serotonin, its precursor and metabolite in Tph2+/+ and Tph2+/− mice.
(A) StressedTph2+/− mice displayed a significantly higher 5-HTP concentration in the amygdala than non-stressed Tph2+/− mice and stressed Tph2+/+ controls. **(B)** In comparison with non-stressed Tph2+/+ mice, non-stressed Tph2+/− mice exhibited decreased 5-HT concentration in the amygdala. **(C)** Stressed Tph2+/+ group showed elevated HIAA concentration in the amygdala, compared to non-stressed controls, and stressedTph2+/− mice exhibited a significant increase of this parameter in comparison with non-stressed Tph2+/− mice. **(D)** Stressed Tph2+/+ mice displayed significantly higher 5-HTP concentration in the prefrontal cortex than non-stressed control and stressed Tph2+/− mice. **(E)** In comparison with non-stressed Tph2+/+, stressed Tph2+/+ group demonstrated increased 5-HT concentration in the prefrontal cortex. **(F)**There were no significant differences between groups in HIAA concentration in the prefrontal cortex. **(G)** Stressed Tph2+/+ mice showed elevated 5-HTP concentration in the hippocampus, compared to stressed Tph2+/− mice. **(H)** No significant differences were found between groups in hippocampal concentrations of 5-HT. **(I)** Stressed Tph2+/+ demonstrated augmented HIAA concentration in the hippocampus in comparison with non-stressed Tph2+/+ and stressed Tph2+/− mice. **(J)** Stressed Tph2+/+ animals showed higher 5-HTP concentration in the dorsal raphe than stressed Tph2+/− mice. **(K)** No significant differences were found between the groups in the 5-HT concentration **(L)** and in the HIAA concentration in dorsal raphe. **(M)** Stressed Tph2+/+ mice displayed a higher 5-HTP concentration, **(N)** a 5-HT concentration and **(O)** a HIAA concentration in the striatum than non-stressed Tph2+/+ mice. 5-HTP and HIAA concentrations were increased in the striatum of stressed Tph2+/+ in comparison with stressed Tph2+/− mice. Kruskal-Wallis test, two-way ANOVA and post hoc Tukey's test or Bonferroni test. Tph2+/+ No stress, n=7, Tph2+/+ Stress, n=7, Tph2+/− No stress, n=8, Tph2+/− Stress, n=7. Bars are Mean ± SEM (C,I,J,K,L,M,O) or Median with interquartile range (A,B,D,E,G,H,F,N).

significantly different between the groups ($H=12.8$, $p=0.0051$; Kruskal-Wallis test); in comparison with respective non-stressed groups, this measure was increased in stressed Tph2+/+ mice ($p=0.0017$) and decreased in stressed Tph2+/− mice ($p=0.0076$; Fig. 5D). ANOVA revealed a significant effect of stress, genotype and their interaction for hippocampal GluA2 mRNA concentration ($F_{3,28}=6.21$, $p=0.0222$; $F_{3,28}=8.3$, $p=0.0096$ and $F_{3,28}=15.4$, $p=0.0009$). Stressed Tph2+/− mice demonstrated its increase in comparison with mutants and stressed controls ($p=0.0002$ and $p=0.0001$, Bonferroni test, respectively; Fig. 5F). There was significant effect of stress on GluA2 mRNA concentration in the dorsal raphe ($F_{3,28}=9.25$, $p=0.0067$, two-way ANOVA), but no significant effect of genotype ($F_{3,28}=1.73$, $p=0.2040$) and stress and genotype interaction ($F_{3,28}=1.36$, $p=0.2632$; Fig. 5H). ANOVA revealed no significant stress effect for GluA2 mRNA concentration in the striatum ($F_{3,28}=0.101$, $p=0.7538$, two-way ANOVA) or interaction ($F_{3,28}=1.36$, $p=0.2632$), but there was a significant genotype effect ($F_{3,28}=5.2$, $p=0.0398$). GluA2 expression was significantly higher in the striatum of stressed Tph2+/− mice than in stressed Tph2+/+ controls ($p=0.0411$, Bonferroni test; Fig. 5J). No further group differences were observed.

For 5-HT1a and 5-HT2a receptors in the prefrontal cortex, there was no significant effect of stress ($F_{3,28}=0.37$, $p=0.55$ and $F_{3,28}=0.781$, $p=0.3879$, respectively, two-way ANOVA), genotype ($F_{3,28}=0.271$, $p=0.8711$ and $F_{3,28}=0.511$, $p=0.4834$, respectively) or an interaction ($F_{3,28}=0.0271$, $p=0.8711$ and $F_{3,28}=0.121$, $p=0.7321$, respectively, Suppl.Fig. 2A,B). No group differences were found.

Thus, stressed Tph2+/− mice had an overall decreased expression of the 5-HT6 receptor and an increased expression of the AMPA receptor GluA2 subunit, which in majority of cases was not found in the stressed Tph2+/+ animals. At the same time, the expression of 5-HT1a and 5-HT2a receptors was unaltered.

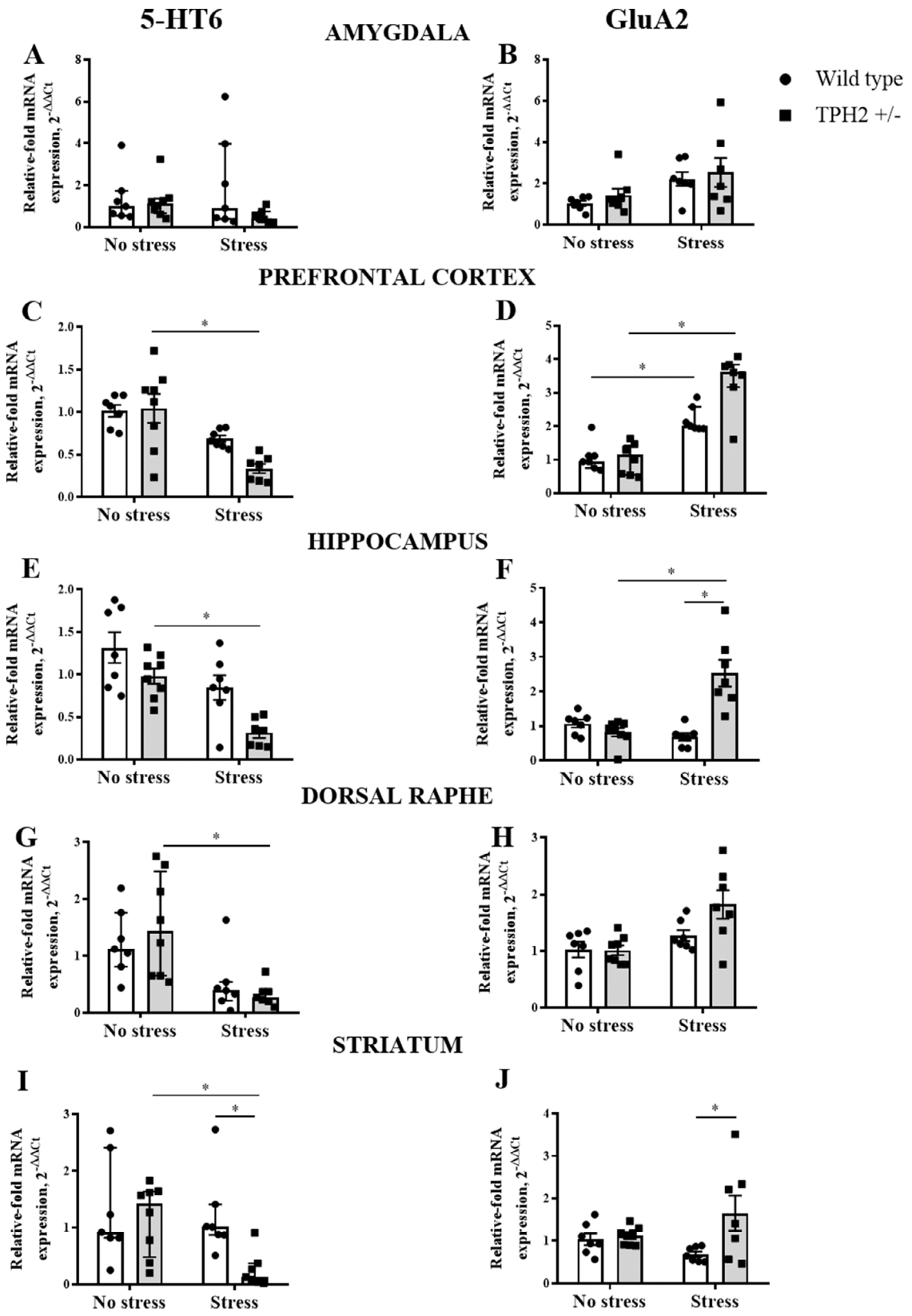
3.5. Altered gene expression of GSK-3β and c-fos in Tph2+/− and Tph2+/+ mice exposed to predator stress

Group comparison has revealed significant differences in GSK-3β mRNA concentration in the amygdala ($H=7.254$, $p=0.0498$; Kruskal-Wallis test). Both stressed Tph2+/+ and Tph2+/− mice displayed elevated GSK-3β mRNA levels in comparison with respective non-stressed groups ($p=0.0445$, $p=0.0117$, respectively; Suppl.Fig. 1A). Stress significantly affected GSK-3β mRNA concentration in the prefrontal cortex ($F_{3,28}=20.4$, $p=0.0002$, two-way ANOVA), while no significant effects of genotype ($F_{3,28}=0.381$, $p=0.5446$) and stress and genotype interaction ($F_{3,28}=0.0031$, $p=0.9568$) were found. Both stressed Tph2+/+ and Tph2+/− mice displayed elevated GSK-3β mRNA levels in comparison with their respective non-stressed groups ($p=0.0124$ and $p=0.0083$, respectively; Suppl.Fig. 1C). For hippocampal GSK-3β mRNA concentration, a two-way ANOVA showed no significant effect of stress ($F_{3,28}=0.293$, $p=0.5943$) genotype ($F_{3,28}=0.55$, $p=0.4673$) or their interaction ($F_{3,28}=1.16$, $p=0.295$; Suppl.Fig. 1E). Significant effect of stress for GSK-3β mRNA concentration in the dorsal raphe ($F_{3,28}=5.74$, $p=0.0271$, two-way ANOVA), but no significant effects of genotype ($F_{3,28}=0.0519$, $p=0.8222$) and stress and genotype interaction ($F_{3,28}=0.0228$, $p=0.8815$; Suppl.Fig. 1G) were observed. Two-way ANOVA revealed no significant effect for GSK-3β mRNA concentration in the striatum of stress ($F_{3,28}=0.482$, $p=0.496$), genotype ($F_{3,28}=2.36$, $p=0.1411$) and their interaction ($F_{3,28}=0.0736$, $p=0.7891$; Suppl.Fig. 1I). No further group differences were found.

Stress significantly affected c-fos mRNA concentration in the amygdala ($F_{3,28}=5.44$, $p=0.038$, two-way ANOVA), however, there were no significant effects of genotype ($F_{3,28}=0.199$, $p=0.6604$) and stress and genotype interaction ($F_{3,28}=0.11$, $p=0.7433$). Both stressed Tph2+/+ and stressed Tph2+/− mice showed higher values of this measure than in their respective non-stressed groups ($p=0.0312$ and $p=0.0457$, respectively, Tukey's test; Suppl.Fig. 1B). A significant effect of stress was shown for c-fos mRNA concentration in the prefrontal cortex ($F_{3,28}=19.2$, $p=0.0003$), that was not found for genotype ($F_{3,28}=0.0584$, $p=0.8116$) and no stress and genotype interaction ($F_{3,28}=0.00326$, $p=0.9551$). Both stressed control and mutant groups showed increases in this measure` in comparison with respective control groups ($p=0.0027$ and $p=0.0005$, respectively, Bonferroni test; Suppl.Fig. 1D). Analysis with a Kruskal-Wallis test showed no significant differences between groups for c-fos mRNA concentration in the hippocampus ($H=4.938$, $p=0.1764$; Suppl.file1F). c-fos mRNA concentration in the dorsal raphe and striatum was not effected by stress ($F_{3,28}=1.13$, $p=0.3003$ and $F_{3,28}=0.00122$, $p=0.9725$, respectively; two-way ANOVA), genotype ($F_{3,28}=0.0675$, $p=0.7978$ and $F_{3,28}=0.567$, $p=0.4607$), and there was no interaction ($F_{3,28}=1.13$, $p=0.3003$ and $F_{3,28}=0.0775$, $p=0.3896$; Suppl.Fig. 1J,H). No further group differences were revealed for c-fos mRNA concentration. Together, these data suggest that the overexpression of GSK-3β and c-fos is unlikely to underlie excessive aggression present in the mutants, as their expression was elevated in all stressed mice regardless of genotype.

4. Discussion

We found that stressed Tph2+/− mice exhibited increased aggressive and dominant-like behaviors compared to naïve Tph2+/− and Tph2+/+ mice. Thus, the interaction of partial TPH2 gene deficit and stress led to pathological aggression reminiscent of the behavioural phenotype of naive Tph2 null mutants. By contrast, measures of aggressive and dominant-like behaviours in Tph2+/+ controls were significantly diminished after stress. Remarkably, maximal expression of aggression in Tph2+/− mice was observed at the beginning of the interaction. Instead, Tph2+/+ mice displayed typical trends of increasing agonistic interactions over the course of successive social contacts. Overall, Tph2+/− mice and Tph2+/+ subjected to a predator stress paradigm displayed opposite changes in aggressive and



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dominant behaviors with distinct dynamics in the expression of the aggressive phenotype.

While the present study supports our hypothesis that stress would induce aggressiveness in Tph2+/−, we did not detect a further decrease in brain 5-HT in stressed Tph2+/− compared to non-stressed Tph2−/− mice. This was probably due to a ceiling effect of altered 5-HT concentrations in these animals, since 5-HT concentration is significantly decreased in Tph2+/− in normal conditions. There was a

Fig. 5. Stress-induced changes of the expression of 5-HT6 receptor and AMPA receptor GluA2 subunit in Tph2+/+ and Tph2+/− mice.
No significant differences were found between groups in **(A)** 5-HT6 mRNA concentrations **(B)** and GluA2 mRNA concentrations in the amygdala. **(C)** Stressed Tph2+/− mice showed significant decrease of 5-HT6 mRNA concentration in the prefrontal cortex in comparison with non-stressed Tph2+/− mice. **(D)** Stressed Tph2+/+ animals had higher 5-HT6 mRNA level in prefrontal cortex than their respective non-stressed group; stressedTph2+/− mice showed a significant increase of 5-HT6 mRNA as compared to non-stressed Tph2+/− mice. **(E)** Stressed Tph2+/− mice displayed lower than non-stressed Tph2+/− mice 5-HT6 mRNA concentrations in the hippocampus. **(F)**In comparison with non-stressed Tph2+/− mice and stressed Tph2+/+ mice, stressed mutantsdemonstrated significantly upregulated GluA2 mRNA concentrations in the hippocampus. **(G)** Stressed Tph2+/− mice demonstrated significantly decreased 5-HT6 mRNA level in dorsal raphe, compared to non-stressed Tph2+/− mice. **(H)** No significant group differences were found in GluA2 mRNA concentrations in the dorsal raphe. **(I)**StressedTph2+/− mice showed a significant increase of this measure in striatum in comparison with non-stressed Tph2+/− mice and stressed Tph2+/+ controls. **(J)**StressedTph2+/− mice displayed significantly higher GluA2 mRNA concentrations than stressed Tph2+/+ controls. *$p < 0.05$, two-way ANOVA and post hoc Tukey's test. 6-9 animals per group were used. Kruskal-Wallis test, two-way ANOVA and post hoc Tukey's test or Bonferroni test. Tph2+/+ No stress, n=7, Tph2+/+ Stress, n=7, Tph2+/− No stress, n=8, Tph2+/− Stress, n=7. Bars are Mean ± SEM (B,C,E,F,H,J) or Median with interquartile range (A,D,G,I).

trend to a reduction in 5-HT levels in the amygdala in Tph2+/+ mice that was not observed in mutants. However, we observed very little reduction in 5-HT concentration in most regions of the brain that were investigated, which is in keeping with other studies that have also reported an absence of changes in 5-HT metabolism in the brain of the Tph2+/− mutants (Angoa-Pérez et al., 2014; Beis et al., 2015). These data indicate that there may be a certain compensatory mechanisms that are adopted during development in Tph2-deficient mice (Waider et al. 2013), which may be similar in Tph2 hypo-expression in patients, who are susceptible to stress, but do not demonstrate behavioral alterations under normal conditions (Mosienko et al., 2012). The Tph2+/+ controls exposed to stress showed significant increases of 5-HT concentrations in the prefrontal cortex and striatum that might be explained by compensatory changes that have been previously reported (Adell et al., 1988), however this was not found in stressed mutant mice. Other studies have shown complex changes in the serotonergic system under conditions of stress, and particularly, in different subpopulations of 5-HT neurons projecting to the prefrontal cortex (Lowry, 2002).

Remarkably, the concentration of the 5-HT precursor 5-HTP was elevated in the amygdala and decreased inother investigated brain regions in the stressed Tph2+/− mice. Contrasting changes were found in conventional mouse strains subjected to stress, for example in C57BL/6J mice, after acute and chronic restraint stress (Browne et al., 2011). The concentration of 5-HIAA was higher in stressed controls, but was unchanged in mutants. Hence, Tph2+/− and Tph2+/+ mice displayed marked differences in changes of 5-HIAA concentration in response to stress. These data are in keeping with studies on mice (Browne et al., 2011) and macaque monkeys, subjected to early life variable foraging demand stress, where increased 5-HIAA concentrations in the cerebrospinal fluid were detected (Coplan et al., 2014). Similar changes for both measures were shown in CF-1 mice exposed to the cold swim stress (Thurmond & Brown, 1984). An increase of urinary 5-HIAA was reported in Sprague-Dawley rats after restraint stress (Kundrotas & Gregg, 1997). In humans, stress, associated with competition, results in increased 5-HIAA excretion in the urine (Helin et al., 1988).

As expression of c-fos was increased in the stress groups of both genotypes, and expression of GSK-3β was elevated in mutant mice regardless of stress experience,these molecular factors are unlikely to mediate excessive aggression in stressed Tph2+/− animals. Moreover, c-fos activation in Tph2+/− was previously found to be unrelated to functional deficits (Waider et al. 2019).

In line with previously reported findings, we showed that stress-induced aggression in Tph2+/− mice was associated with the overexpression of Glu2A and downregulation of 5-HT6 receptor in most of the investigated brain structures (Strekalova et al., 2018; Gorlova et al., 2019; Pavlov et al, 2019). This is also in agreement with previously reported findings in relation to GluA2 protein expression in mice subjected to isolation stress (Shimizu et al., 2016), and anti-anxiety effects of5-HT6 receptor antagonist ADN-1184 in mice and rats (Partyka et al., 2016). Notably, the 5-HT6 receptor is implicated in the regulation of cognition and functionally related with activity of GABAergic neurons (de Jonk & Mørk, 2017), whose functionality is altered in null TPH2 mutants (Waider et al., 2013; Waider et al., 2017). In human studies, an allelic variant of the 5-HT6 gene was shown to be associated with aggressive behavior (Tsai et al., 1999), and 5-HT6 receptor density was significantly decreased in a cohort of patients with Alzheimer's disease that showed psychotic symptoms (Marcos et al., 2008). It is also of note that the expression of 5-HT1a and 5-HT2a, whose change was reported in Tph null mutants (Araragi et al., 2013; Jørgensen et al., 2013), was unaltered in stressed Tph2+/− mice.

Other changes previously found in Tph2 null mutant mice (Waider et al., 2013,; Gutknecht et al., 2012; Angoa-Pérez et al., 2012; Gutknecht et al., 2009; Gutknecht et al., 2015) might underlie stress-induced changes in aggressive behavior of mice partially deficient for TPH and are worthy of investigation. These mechanisms may involve a reduction in the density of GABAergic interneurons in the basolateral amygdala, decreased number of noradrenergic neurons in the locus coeruleus and diminished noradrenalin concentration in many brain regions. Additionally, a reduction in hippocampal dopamine levels, lowered plasma testosterone concentration and altered corticosterone metabolism have been found in Tph2 null mutants, and it would be interesting to investigate these measures in the stressed Tph2+/- mice.

5. Conclusion

Here we report striking differences in aggressive / dominant behaviors between two genotypes exposed to stress. This was accompanied by differential changes of the brain metabolism of 5-HT. Excessive aggression of stressed Tph2+/− mice was associated with the upregulation of AMPA receptor subunit GluA2, c-fos, GSK-3βand a downregulation of 5-HT6 receptor; the majority of these changes were not found in the Tph2+/+ groups. Together, the present study has identified a number of molecular changes that are associated with aggression in a ‘double-hit’ paradigm following the combination of TPH deficiency with stress. These molecular changes are in keeping with molecular changes presents in other animal models of stress induced aggression and highlight their use as possible targets of pharmacological management for excessive aggression.

Declaration of Competing Interest

On behalf of all authors, I would like to state that none of the authors involved in the work have any competing interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jad.2020.04.014.

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